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Thanks,

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Possible Relationship between Vascular Permeability Factors, Endothelial Cells, and Peritumoral Brain Edema

A Neurosurgeon's Perspective

Gregory Richard Criscuolo

I. INTRODUCTION

Cerebral edema is a significant cause of the neurological morbidity associated with malignant brain tumors. Patients afflicted with intracranial neoplasms typically present with progressively worsening headaches, vomiting, blurred vision, double vision, and depressed level of consciousness. All of these clinical features are clearly related to elevated intracranial pressure (ICP), which may rapidly result in a patient's demise if not treated promptly and effectively. The brain edema associated with cerebral tumors is an ultrafiltrate of plasma containing water, electrolytes, and plasma proteins, that emanates from the brain tumor microvasculature. Edema fluid typically infiltrates the white matter surrounding an intracerebral tumor in a diffuse manner, while relatively sparing the adjacent cortex. This excessive accumulation of cerebral interstitial tissue fluid contributes to the distortion of normal intracranial structures, and to elevation of the ICP. As a result, cerebral edema is frequently as culpable for a brain tumor patient's symptoms as the primary intracerebral neoplasm. It is now widely accepted that this excessive fluid accumulation results from a flaw in the integrity of the blood–brain barrier. The blood–brain barrier is both an anatomical and a physiological system which normally regulates the entry and egress of substances between the cerebral interstitial and intravascular compartments. The functional components of the blood–brain barrier occur primarily at the level of the vascular endothelial cell and, to a lesser extent, the basement membrane and astrocytic processes which invest the cerebral microvasculature. Ultrastructural examination of normal brain

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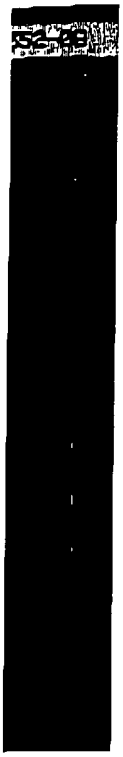
microvessels in comparison to brain tumor microvessels supports the time-honored hypothesis that altered tumor vessels are in fact responsible for the abnormal extravasation of fluid and protein into the interstitial space; thus revealing the vasogenic nature of this process. Despite extensive efforts to study this phenomenon, a focused pathophysiological explanation for these anatomical observations has not yet evolved.

It is recognized that treatment of brain tumor patients with high doses of glucocorticosteroids, such as dexamethasone, results in a remarkable resolution of their ICP-related signs and symptoms. Moreover, the onset of this clinical efficacy is readily apparent within a predictable 12 to 48 hr of initiation of this therapy. This dramatic response is of practical significance as it allows the neurosurgeon, patient, and patient's family additional time prior to performing surgery, to discuss issues related to the disease process, surgical therapy, and perioperative care. Furthermore, patients undergoing brain tumor surgery are well known to enjoy a better surgical outcome if their neurologic deficits are minimized preoperatively. Clinical improvement with dexamethasone appears to correlate with partial resolution of vasogenic brain edema as evidenced by computed tomographic (CT) studies. Notably, the clinical responsiveness of brain edema to dexamethasone has been shown to correlate with cytosolic glucocorticoid-receptor concentrations in several types of intracranial tumor. Nevertheless, the pharmacological basis for this remarkable clinical, physiological, and radiographic response is unknown, and the rationale for glucocorticoid use in this setting has been largely empirical. It would appear, therefore, that further study of the extent and specificity of dexamethasone's actions in the setting of neoplastic brain edema would be worthwhile, as it might reveal novel insight into the very mechanism by which certain types of edema evolve.

II. DEFINITION AND CLASSIFICATION OF BRAIN EDEMA

Brain edema is generally defined as a condition whereby disturbed cerebral homeostatic mechanisms result in an abnormal increase in brain tissue volume that is largely attributable to an increase in water content. Many pathological processes are known to result in brain edema formation. Although the pathophysiological mechanisms are not completely understood, it is apparent that several distinct varieties of cerebral edema exist.[4,48,70] Vasogenic brain edema (type I) is associated with marked alterations of the microvascular elements. This disturbance in the blood–brain barrier results in extravasation of plasmalike fluid into the white matter extracellular space (white matter edema). Vasogenic edema is seen clinically in the setting of brain tumor (Fig. 1), abscess, intracerebral hemorrhage, malignant systemic hypertension, and after prolonged seizure activity. Cytotoxic brain edema (type II) is initially associated with damage to cortical cells resulting in impaired cellular membrane homeostasis and permeability. Intracellular accumulation of sodium and water results in cellular swelling (gray matter edema) at the expense of the interstitial space. Cytotoxic brain edema is seen clinically in the setting of cerebral ischemia or infarction, diabetic coma, hepatic encephalopathy, Reye's syndrome, and pseudotumor cerebri. Interstitial brain edema (type III) refers to an infiltration of cerebrospinal fluid (CSF) into the periventricular white matter. The cerebral interstitial fluid normally communicates freely with the CSF space and a bulk diffusion occurs in a centrifugal fashion. Interstitial brain edema occurs in the setting of acute hydrocephalus



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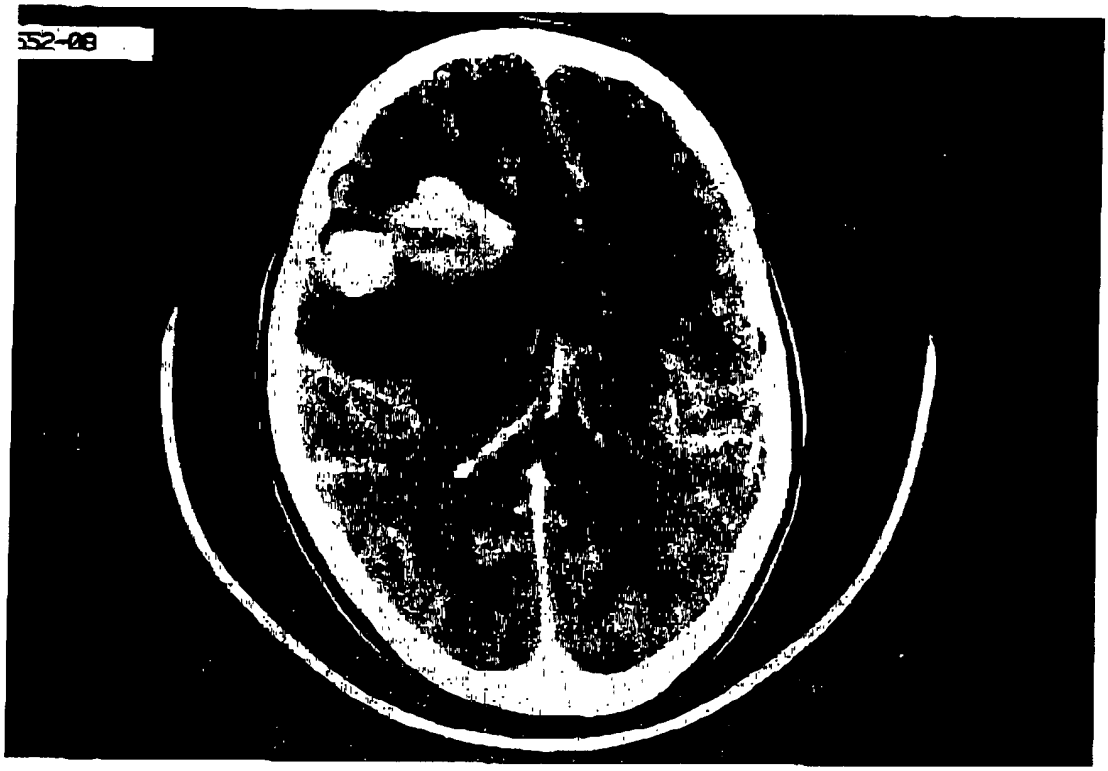

Figure 1. Computer-assisted tomographic brain scan (CT) of a middle-aged person who presented clinically with a 2-week history of progressively worsening headaches, vomiting, double vision, impaired mentation, and left-sided weakness. The multilobular lesion seen within the right frontal lobe was found, at the time of neurosurgical biopsy and excision, to be a highly malignant brain tumor of glial cell origin (glioblastoma multiforme). The brightness of this lesion relates directly to extravasation of an intravenously administered iodinated contrast agent, in areas of frank blood–brain barrier disruption. Note the dark radiolucent region of vasogenic brain edema which arise from within the tumor and characteristically infiltrates the subcortical white matter surrounding the tumor. Cerebral edema appears to be contributing as much to the mass effect and brain distortion as the tumor itself. Within 24 hr of starting high-dose dexamethasone (10 mg i.v. every 4 hr), the patient had returned to normal and was able to undergo an uncomplicated gross total excision of this tumor.

whereby CSF under increased pressure is forced to flow centripetally across the ependymal surface lining the ventricular cavities, and into white matter interstitial space. Blood–brain barrier function is not altered. Osmotic/hydrostatic brain edema (type IV) occurs when a sudden disturbance in Starling's equilibrium results in the development of an osmotic gradient between plasma and cerebral tissue. Initially, the edema consists largely of free water without electrolytes or plasma proteins. The brain eventually loses electrolytes without gaining significant quantities of water. Clinical impairment is more likely related to cellular potassium loss and hyponatremia, as elevated ICP is not a consistent association. Osmotic or hydrostatic brain edema occurs with primary water intoxication, rapid hemodialysis, and the inappropriate secretion of antidiuretic hormone (SIADH). This classification of cerebral edema can be quite sound and useful provided one recognizes its limitations. For instance, it is well recognized that disparate types of brain edema can coexist. For instance, both vasogenic and cytotoxic edema may occur in certain settings

depending upon the nature and severity of the initial insult. Furthermore, if one considers the variety of distinct pathophysiological events that may result in expression of the same pattern of edema, a lack of specificity becomes self-evident. Nevertheless, this template rightfully remains the standard for all discussions about edema ultrastructure, pathology, and physiology.

III. BRAIN TUMORS AND TUMOR-ASSOCIATED CEREBRAL EDEMA

A. Survey of Human Brain Tumors

Tumors of the central nervous system (CNS) pose a major challenge to the field of oncology. They continue to be associated with a high degree of morbidity and mortality, despite advances in neurosurgery, radiotherapy, and chemotherapy.[3,8,28,34,40,51,81,83,100] Primary tumors of the CNS account for approximately 10% of all malignancy. Furthermore, cancer is second only to trauma as a cause of death in childhood, and brain tumors represent the second most common childhood malignancy. About 20,000 new primary brain tumors and an additional 15,000 secondary or metastatic brain tumors are diagnosed each year in the United States. In fact, intracranial neoplasms account for 2% of all cancer-related deaths.

Approximately 40% of all CNS tumors are primary tumors derived from neuroectodermal supporting cells (glial cells) such as astrocytes, oligodendrocytes, and ependymal cells. Astrocytomas comprise 60 to 70% of glial cell tumors (gliomas). Several attempts have been made to create a practical grading system for these lesions by correlating histological features with their biological behavior. One of the more commonly employed systems assigns a numerical grade from I through IV. Grade I and grade II astrocytomas are generally considered histologically and biologically benign tumors characterized by variable degrees of hypercellularity, and relatively prolonged survival after treatment with surgical excision or radiation therapy. Grade III (anaplastic astrocytoma) and grade IV (glioblastoma multiforme) astrocytomas display malignant cellular features such as extreme hypercellularity, pleomorphism, increased nucleocytoplasmic ratio, mitotic figures, nuclear hyperchromatism, multinucleation, and bizarre giant cell forms. Malignant stromal changes such as necrosis, pseudopalisading, edema fluid accumulation, and microvascular endothelial proliferation occur almost exclusively in the most highly malignant tumor varieties (glioblastoma multiforme).

Survival in patients with malignant gliomas is poor and ranges from 3 to 6 months untreated. Combined surgical excision, radiation, and chemotherapy have improved survival but mortality still approaches 95 to 100% at 5 years postdiagnosis. A peculiarity of primary brain tumors is the rarity with which they metastasize beyond the CNS. In most instances, malignant brain tumors are locally invasive, and therefore limit their growth to the fixed confines of the intracranial cavity. Recurrences after surgical excision and radiation therapy are most likely to occur at the site of the original tumor. Certain tumors demonstrate a predilection for seeding malignant cells into the subarachnoid space throughout the leptomeninges and spinal cord along CSF pathways (medulloblastoma, ependymoma, pinoblastoma, ependymoblastoma). Spread of a brain tumor in this fashion would naturally pose additional diagnostic and therapeutic challenges.

Brain tumors most likely to be associated with clinical and CT-evident vasogenic cerebral edema include most primary malignant tumors (anaplastic astrocytoma and glioblastoma multiforme), many secondary malignant tumors (metastases from lung, breast, renal, thyroid, and colon cancer, and malignant melanoma), and certain benign tumors (meningioma). Their management is complicated by the presence of peritumoral brain edema, which frequently limits a patient's tolerance for essential brain tumor therapies (surgery, radiotherapy, and chemotherapy) that may themselves initially contribute to cerebral swelling. Furthermore, the risk of a poor outcome after neurosurgical excision of a brain tumor is increased substantially in the setting of severe, symptomatic brain edema. For these reasons, an expanded understanding of the pathophysiology of peritumoral brain edema is essential to the effective treatment of patients with intracranial tumors.

B. The Blood–Brain and Blood–CSF Barriers

The concept of the blood–brain barrier was initially put forth by Paul Ehrlich, a 19th century German bacteriologist who noted that intravascular injection of vital dyes stained all organs except the brain. His interpretation, that this related to a differential organ affinity for the dye, was later proven incorrect when in 1913 a student of his, Edwin E. Goldmann, showed that injection of trypan blue into the CSF readily stained the brain but failed to enter the bloodstream. The blood–brain barrier is now recognized to be a complex, highly selective anatomical and physiological barrier, which regulates the entry and exit of cerebral nutrients and biologically important substances necessary for the maintenance of cerebral metabolism and neuronal activity.[4,13,35,54] Although Goldmann was the first to postulate that brain capillaries were the anatomic basis for the blood–brain barrier, direct confirmation did not occur until the advent of electron microscopy in the 1950s, which demonstrated the continuous tight junctions between adjoining endothelial cells.

Endothelial cells of the cerebral vasculature display several features attesting to their unique participation in blood–brain barrier function. The single most important feature is the high-resistance, pentalaminar tight junctions that fuse endothelial cells together in a continuous layer, and effectively form a physical barrier between circulating molecules and cells, and the cerebral interstitial space. Additional specialized features include: continuous capillary basement membranes, paucity of endothelial micropinocytotic activity, abundance of endothelial cell mitochondria, and absence of endothelial membrane fenestrations.[9–13,20,36,64] Therefore, virtually all macromolecules are excluded by the blood–brain barrier, and diffusion of substances into the brain largely depends upon physical characteristics such as molecular size, electrostatic charge, and lipophilicity. Amino acids, glucose, biogenic amines, and other essential brain nutrients gain entry by a complex system of membrane transporters. The various entry mechanisms may involve active transport (energy-requiring), facilitated transport (not energy-requiring), and enzymatic modification of a molecule prior to entry. The blood–CSF barrier is an analogous but distinct entity whose function is governed by the selective secretory activity of the choroid plexus epithelium. Together, the blood–brain and blood–CSF barriers regulate the composition of the cerebral interstitial fluid and CSF within well-defined limits. Disturbances in any of the components of the blood–brain or blood–CSF barriers, if significant, will result in cerebral edema.

C. Ultrastructure of Brain Edema and the Tumor Microvasculature

Prior reports have described the increased permeability characteristic of the microvasculature within primary and metastatic malignant brain tumors, as well as certain benign tumors.[55–57] Early studies focused primarily on the morphology of cerebral edema, and the altered vascular ultrastructure of tumor-associated and peritumoral blood vessels. Vasogenic brain edema was characterized by infiltration of the white matter interstitial spaces by an ultrafiltrate of plasma.[4,13,48,70] It has been postulated that the specialized features of normal brain endothelial cells result from contact with normal brain tissue.[5,9,10] As the latter consists largely of astrocytes whose cytoplasmic processes enmesh these microvessels, it would follow that the vastly altered milieu inherent in brain tumors, where microvessels develop among abnormal astrocytes (gliomas), or in their absence (metastatic tumors), might account for a dedifferentiation of brain endothelial cells into a less specialized phenotype. One can further speculate that microvessels growing into minimally altered environments might retain some blood–brain barrier features (low-grade astrocytomas associated with minimal or no brain edema), whereas the microvasculature of highly anaplastic gliomas retains little or no semblance to normal brain microvessels (associated with extensive brain edema).

Several features of tumor-associated vascular endothelium, such as widened intracellular junctions, discontinuous tight junctions, membrane fenestrations, noncontiguous basement membranes, active micropinocytosis, and paucity of mitochondria, sharply contrasted with the normal architecture of the blood–brain barrier.[1,12,13,36,55–57,66,94,102,103] However, many of these heterotypical cellular features of brain tumor microvessels typify the endothelium lining normal peripheral vasculature (not involving the blood–brain barrier). While the peripheral vasculature is not inherently permeable to macromolecules, it does appear to be exquisitely sensitive to permeability induction by physiologically occurring substances such as histamine, bradykinin, serotonin, and prostaglandin.[52,62,65] Furthermore, normal cerebral blood vessels appear totally unable to respond to these potent mediators of microvascular extravasation. Therefore, although it appears unlikely that the absence of continuous pentalaminar tight junctions would solely account for the abnormal permeability of brain tumor microvessels, alteration of this critical component of blood–brain barrier may at once render brain tumor microvessels similar to the peripheral microvasculature in anatomical form, and physiological reactivity to permeability-inducing substances.

D. Current Clinical Management of Peritumoral Brain Edema

Patients with brain tumors present most often with clinical manifestations of elevated ICP. They typically complain of headache, nausea, vomiting, drowsiness, and double vision. Seizures and focal neurologic deficits are also commonly present, and their precise pattern, distribution, and severity are dictated largely by the location and size of the tumor and the extent of the surrounding cerebral edema (Fig. 1). Progressive elevation of the ICP will result in clinical progression to stupor and coma. Ultimately, intracranial hypertension leads to cerebral herniation with consequent fatal brain stem compression. In this regard, peritumoral vasogenic cerebral edema deserves reemphasis as a frequent and important accompaniment of intracranial tumors. The combined effects of rapid tumor growth and

cerebral swelling within the confines of the cranial vault, dictate the rapidly progressive clinical decline observed in patients with malignant brain tumors. Methods of acutely managing these patients include tumor excision (surgical cytoreduction), fluid restriction, diuresis with intravenous furosemide and mannitol, high-dose intravenous dexamethasone, and ventricular CSF drainage. Tumor excision addresses the tumor mass and its vascular bed directly, and as a consequence, eliminates the source of vasogenic edema fluid. Fluid restriction and diuretics decrease ICP by contracting the intravascular space and facilitating the egress of edema fluid from the cerebral interstitial space and into the vascular compartment. Ventricular CSF drainage lowers ICP by reducing the volume of the cranial contents; however, it is most specific in the setting of hydrocephalus related to tumor obstructing CSF outflow, and may be quite dangerous in the presence of a focal mass lesion with impending brain herniation. Glucocorticoids such as dexamethasone are remarkably effective in reducing the neurological deficits and intracranial hypertension associated with malignant brain tumors.[15,29,42,54,69] Their efficacy is not the result of tumor cytolysis and CT data indicate a direct action upon the peritumoral edema.[33,49,104,106] In fact, myriad studies have clearly shown neoplastic vasogenic brain edema to be the only type of brain edema that responds to glucocorticoid therapy (Fig. 2).[23,38,67] Once a patient has been stabilized, the brain tumor may be more safely neurosurgically excised. The need for additional therapy postoperatively is largely governed by the extent of tumor resection and histopathological diagnosis. Definitive treatment plans are proposed only after consultation between the neurosurgeon, medical oncologist, and radiation therapist. It is not unusual, however, for patients with residual tumor and edema (i.e., those in greatest need of further therapy) to have adjunctive radiation or chemotherapy interrupted as a result of exacerbation of the edemagenic component. Once again, dexamethasone, employed in even higher doses than used initially, may control the brain edema sufficiently to allow resumption of adjunctive therapy.

IV. MEDIATORS OF MICROVASCULAR PERMEABILITY: A CHRONOLOGICAL SYNOPSIS

A. Introduction

The microvasculature of many solid tumors exhibits increased permeability in comparison to normal tissues. Certain pathological reactions associated with neoplastic growth are likely to result from permeability induction by tumor cells. Clinical manifestations of this process include decreases in serum albumin, production of malignant effusions and ascites, tissue edema, and paraneoplastic arthropathy.[78,86,93] Neoplastic vasogenic brain edema likely derives from a physiological alteration in the blood–brain barrier that is manifested by ultrastructural changes in tumor microvessels, and the extravasation of water, salts, and serum proteins into the peritumoral white matter.[13,48,55–57] Several biochemical mediators have been implicated in the pathogenesis of brain edema. Substances such as histamine, serotonin, bradykinin, glutamic acid, polyamines, leukokinins, lymphokines, prostaglandins, thromboxane, prostacyclin, kallidin, lymphocyte permeability factors, kallikrein, and plasminogen activator are potent inducers of microvascular permeability associated with allergic, traumatic, ischemic, infectious, and inflammatory conditions.[6,16,21,24,52,58,59,68,77,91,95,96]

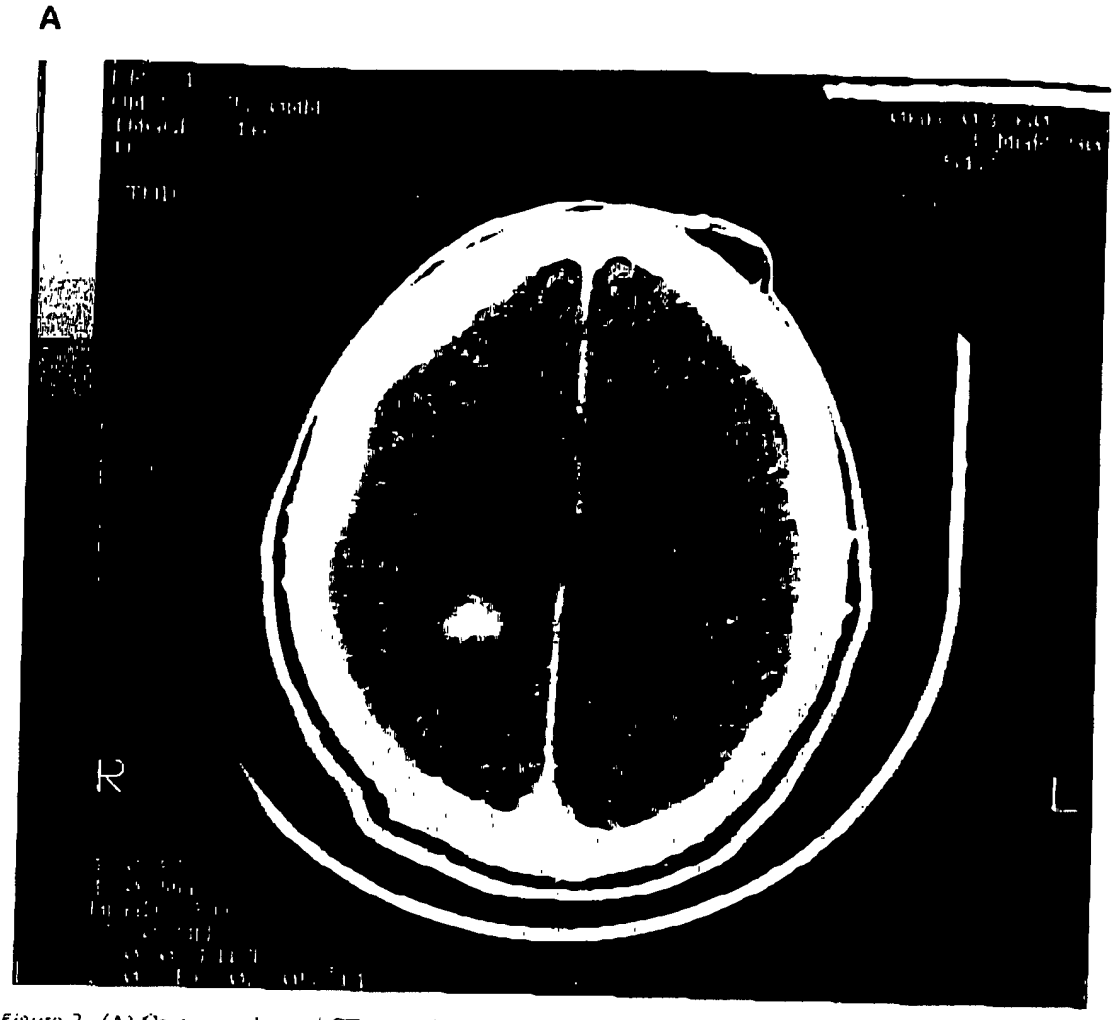

Figure 2. (A) Contrast-enhanced CT scan of a 60-year-old person with lung cancer and a history of progressive left-sided weakness and headache. A small, discrete, brightly enhancing lesion is evident in the right parietal lobe. Equally apparent is the extensive low-attenuation region surrounding this lesion. These findings are quite consistent with a metastatic brain tumor which in this instance is derived from a lung primary. The patient was started on high-dose dexamethasone therapy prior to craniotomy for tumor excision. (B) Contrast-enhanced CT scan performed 72 hr after initiation of dexamethasone therapy. The patient was neurologically normal at this time. The complete absence of contrast enhancement represents a dexamethasone-induced increase in blood–brain barrier integrity. Actual resolution of the edema will occur in a more delayed fashion. The patient underwent a successful total excision of this tumor and was neurologically intact postoperatively.

Furthermore, attention has recently been focused on the potential role of oxygen free radicals in the formation of traumatic and peritumoral brain edema.[43,44]

B. Initial Studies of Tumor-Derived Vascular Permeability Factors

A series of proteinaceous vascular permeability factors (VPFs) have stirred considerable interest in the past decade.[14,17–19,47,50,53,68,76,78–80,86] The existence of VPF was first

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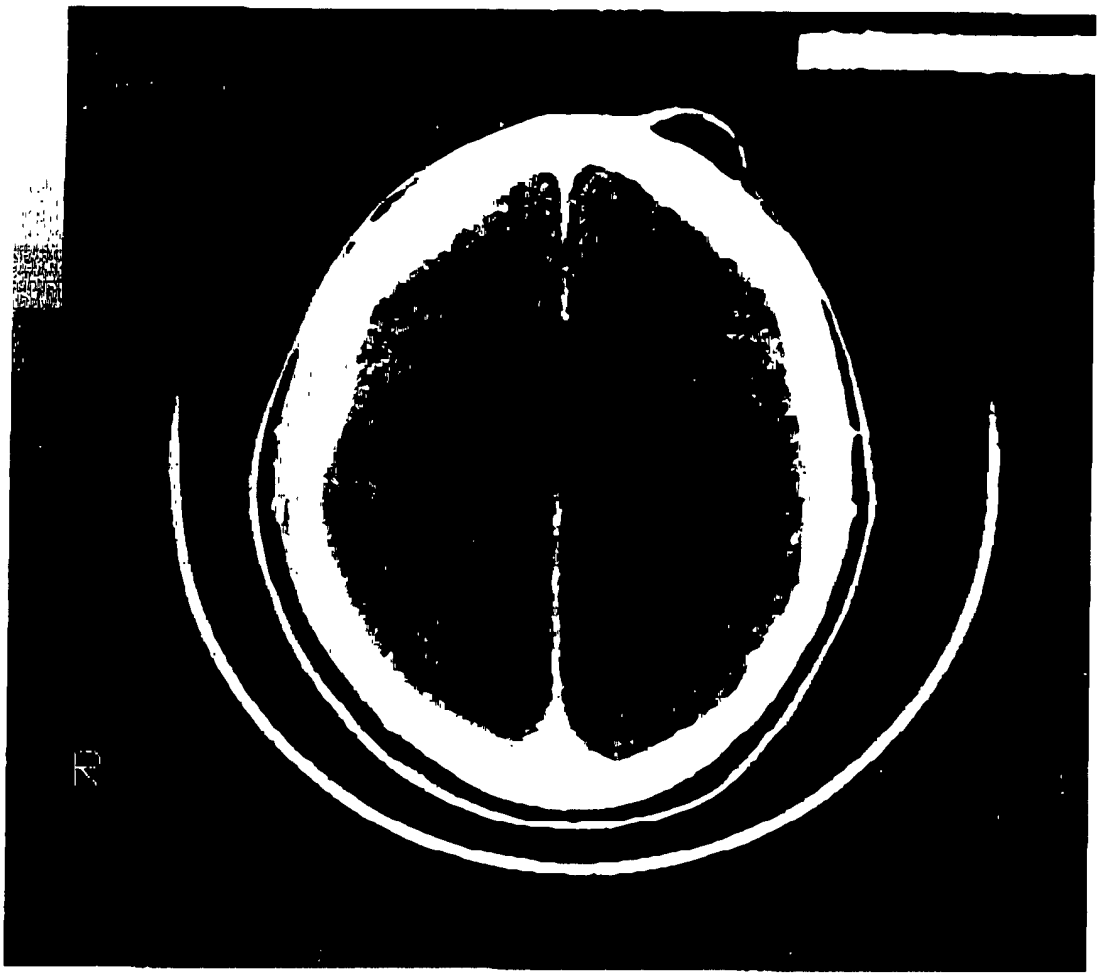

Figure 2. (continued)

recognized to be expressed by a guinea pig hepatocarcinoma (line 10) which promoted the accumulation of ascites fluid.[78] This factor (gVPF) was found to be a 34- to 42-kDa basic protein with a strong affinity for immobilized heparin, and a permeability-inducing potential three orders of magnitude greater than histamine (when compared on a molar basis). The presence of gVPF activity has been routinely determined by the Miles assay, a quantitative bioassay for induction of microvascular permeability.[62] Investigators found that gVPF-induced microvascular extravasation was rapid in onset (1 min) and of short duration (20 min), suggesting a direct action upon the microvascular endothelial cell. Furthermore, gVPF activity induced a period of desensitization whereby microvessels previously exposed to this substance became temporarily refractory (for less than 30 min) to further permeability induction. Light and electron microscopy showed that gVPF did not induce endothelial cell damage or mast cell degranulation when injected into the guinea pig subcutaneous microvasculature. Furthermore, intravascular injection of colloidal carbon

resulted in the labeling of postcapillary venules, thus supporting an action of gVPF upon these critical microvessels.[58,59,85] Rabbit-derived immunoglobulin (polyclonal IgG) raised to partially purified guinea pig hepatocarcinoma-derived gVPF neutralized essentially all permeability-inducing activity present in tumor ascites fluid and tumor conditioned medium from that cell line. In addition, VPF activity from guinea pig fibrosarcoma (line 104 C1), and Walker rat carcinoma lines was similarly inactivated by anti-gVPF antibody. It was suggested that secretion of permeability-increasing activity (VPF) may be a common feature of tumor cells accounting for the abnormal accumulation of fluid associated with neoplasms.[78,79]

Soon thereafter, a tumor-derived capillary endothelial cell growth factor was identified and purified from rat chondrosarcoma extracellular matrix.[84] This 18-kDa cationic polypeptide had a marked affinity for heparin, and therefore differed from platelet-derived growth factor (PDGF) and epidermal growth factor (EGF). Nanogram quantities of this substance stimulated both proliferation and mobilization of capillary endothelial cells in culture; activities thought to be key components of angiogenesis induction. Other studies showed that while heparin potentiated tumor-induced angiogenesis on the chorioallantoic membrane, angiogenesis was inhibited when both heparin and cortisone were administered simultaneously. Thus, glucocorticoids were once again identified as having an inhibitory action on a specific process essential to the biology of tumor growth and metastasis.[26,27] At this point, investigators interested in factors acting directly upon endothelial cells diverged into two groups: those studying tumor angiogenesis factors (TAFs) and other endothelial, fibroblast, and smooth muscle cell mitogens; and those investigators studying VPFs.[14,18,19,26,27,41,78–80,84,88,101] Interest in these heparin binding molecules has only recently reconverged with the publication of the amino acid sequence of a VPF molecule exhibiting both permeability-inducing and angiogenic mitogen capabilities.[17,47,50]

Partial purification and characterization of a human colon adenocarcinoma-derived VPF (line HT-29) showed it to be a 45-kDa acidic protein which lacked a specific affinity for heparin.[53] Like guinea pig hepatocarcinoma gVPF, HT-29 permeability-inducing activity was not inhibited by antihistamines, antikinins, pepstatin A, or indomethacin. Several other human tumor lines have since been tested for VPF activity. One study demonstrated significant VPF expression by human osteogenic sarcoma, bladder carcinoma, cervical carcinoma, and fibrosarcoma. VPF activity from these tumors comigrated nearly identically with guinea pig line 10 gVPF (34 to 42 kDa) and was neutralized by antibodies raised to line 10 VPF. Furthermore, a VPF expressed by two human tumorigenic cell lines was also characterized by heparin affinity and a size of 38 kDa.[79] Demonstration of the highly conserved nature of VPF molecules across species lines suggested a broader purpose for VPF-mediated permeability induction by tumors (i.e., extravasation of nutrients to support tumor growth in the extracellular matrix).[2,26,27,79]

C. Identification of Human Brain Tumor VPF[14,18,19]

Serum-free conditioned media derived from cultures of primary human malignant brain tumors (anaplastic astrocytoma and glioblastoma multiforme) have been shown to contain a factor that induces microvascular extravasation of fluid and protein in the Miles permeability bioassay.[14] VPF activity was not expressed by cultured human fibroblasts,

meningioma cells, or several lines of low-grade astrocytomas (gliomas not usually associated with CT-evident brain edema); however, it was strongly expressed by a number of rapidly growing malignant gliomas (associated with CT-evident edema). Human glioma-derived vascular permeability factor (HG-VPF) appears to differ from commonly recognized mediators of vascular permeability. Early studies did, however, demonstrate several similarities to guinea pig line 10 hepatocarcinoma-derived gVPF. HG-VPF is a 41- to 56-kDa basic cationic polypeptide, with an avid affinity for heparin.[18,19] Like gVPF, the time course of permeability induction by HG-VPF is rapid and transient suggesting a direct action upon endothelial cells. As in previous gVPF studies, prior exposure to HG-VPF results in a period of refractoriness, during which further induction of microvascular extravasation by HG-VPF does not occur. Expression of HG-VPF activity is inhibited by incubation of glioma cell cultures with cycloheximide or dexamethasone. Finally, a polyclonal antibody raised to gVPF completely inactivated HG-VPF activity and further supported homology between these substances.[14]

In order to eliminate variations in HG-VPF expression associated with use of several different tumor lines, serum-free conditioned media derived from low-passage cultures of a single cloned human malignant glioma line (U251) were employed for subsequent HG-VPF studies. A partially purified U251 glioma protein product containing HG-VPF has been shown to induce rapid and reversible elevations in cytosolic calcium in several types of cultured endothelial cells.[19] The HG-VPF-induced intracellular calcium ion changes exhibited dose-responsiveness and were inhibited by nonspecific calcium channel blockers (lithium, cobalt, manganese, and lanthanum ions) suggesting that influx of extracellular calcium ions was responsible for the observed cytosolic calcium transients. Reexposure of endothelial cells to the HG-VPF stimulus failed to produce a second calcium ion transient, suggesting that a period of refractoriness similar to that observed *in vivo* had been induced by the initial HG-VPF exposure. Moreover, HG-VPF-induced calcium changes were completely inhibited in endothelial cells previously exposed to dexamethasone.

D. Purification and Sequencing of Human VPF[17,47,78–80]

A VPF has recently been purified to homogeneity from serum-free conditioned medium of human histiocytic lymphoma cell line U937.[17,47,80] The cDNA sequence of human VPF (hVPF) from this line was shown to code for a 189-amino-acid 38- to 40-kDa polypeptide with two identical 20- to 24-kDa subunits that became evident on reducing gels (SDS–PAGE). Available data suggest that native hVPF is a dimer composed of disulfide-linked subunits, each with the same NH_2-terminal amino acid sequence. The NH_2-terminal region of the predicted amino acid sequence of U937 (human) hVPF was found to be 78% identical to the analogous region of line 10 (guinea pig) VPF. The hVPF molecule also bore some similarities in structure to the B chain of platelet-derived growth factor (PDGF-B),[73] as well as several PDGF/v-*sis* oncogene-related proteins. Similarly, a heparin-binding vascular endothelial cell growth factor (VEGF) has recently been purified from conditioned media derived from bovine pituitary folliculostellate cells and phorbol ester-activated human (HL60) promyelocytic leukemia cells.[50] The cDNA sequence of human VEGF is similar to that of hVPF except for an additional 24 amino acids in the hVPF sequence. VPFs with similar immuno-cross-reactivity have been detected in conditioned media derived

from a variety of human and rodent tumor cell lines. In addition to its permeability-inducing activity, hVPF also appears to specifically stimulate endothelial cell growth and angiogenesis.[17,47,50,80] The hVPF molecule did not stimulate [^{3}H]thymidine incorporation or promote growth of other nonendothelial cell types. This feature distinguished it from several other endothelial cell growth factors, such as the heparin-binding fibroblast growth factors (FGFs). which promote growth and replication in nonendothelial, as well as endothelial cell lines. Conversely, several endothelial cell growth factors (EGF, bFGF, aFGF, TGF, IL-1, TNF, and PDGF) failed to exhibit VPF-like permeability-inducing activity when tested in the Miles assay. Other studies have confirmed the permeability-inducing activity of IL-2 but this protein bears no size similarity or sequence homology to the hVPF molecule. [^{125}I]-hVPF was shown to bind specifically and with high affinity to endothelial cells *in vitro*, and could be chemically cross-linked to a high-molecular-weight cell surface receptor, thus suggesting a specific site of interaction between hVPF and the vascular endothelial cell. Complex formation was blocked by excess unlabeled hVPF and anti-hVPF serum, but not by the addition of excess quantities of the aforementioned growth factors. Permeability induction in the Miles assay was observed with as little as 1 nM (8 ng) hVPF.

V. BIOCHEMICAL AND PHYSIOLOGICAL STUDIES OF HUMAN BRAIN-TUMOR VPF

A. HG-VPF Activity, Expression, and Behavior in the Miles Assay[14]

Serum-free conditioned medium from low-passage confluent monolayer cell cultures of human malignant astrocytoma lines evoked macromolecular extravasation (quantitated by measurement of [^{125}I]-BSA accumulation) in the Miles cutaneous microvascular permeability assay.[14] Intradermal injection of this factor results in a permeability change with a rapid onset at 1 to 5 min, a peak at 5 to 15 min, and reversibility by 20 to 30 min. In addition, there is a characteristic period of desensitization or tachyphylaxis whereby injection of HG-VPF into previously exposed sites results in no further induction of permeability. Conditioned medium from benign astrocytoma, meningioma, and fibroblasts demonstrated no significant VPF activity. Fluid aspirated from a cystic glioblastoma contained very high HG-VPF activity, whereas no activity was evident in samples of CSF from a normal volunteer, a patient with a sacral chordoma, or a patient with a malignant cerebral glioma (Table 1). HG-VPF activity increased as the duration of culture incubation was lengthened (Fig. 3).

B. Characterization of HG-VPF[18]

HG-VPF is an acid-stable heat-labile 41- to 56-kDa polypeptide, which is hydrophobic and positively charged under physiological conditions.[18] HG-VPF activity was abolished by treatment with trypsin or pepsin, but was unaffected by ribonuclease A, chondroitinase A,B,C. hyaluronidase, and lipase. Similarly, HG-VPF activity is not inhibited by soybean trypsin inhibitor or hexadimethrine (both known antagonists of tissue plasminogen activator, Hageman factor, and serum kallikrein); or aprotinin (an antagonist of both plasmin and tissue kallikrein); or phenylmethanesulfonyl fluoride [a serine esterase (elastase) inhibitor];

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Table 1. Expression of Vascular Permeability Factor (VPF) Activity[a]

Source of test sample	VPF activity
Experiment A	
Control (DMEM)	2.4 ± 4.0
Fibroblast	7.4 ± 4.3
Meningioma 1	5.9 ± 3.2
Meningioma 2	7.7 ± 4.0
Astrocytoma	15.2 ± 5.8
Glioblastoma 1	25.7 ± 3.9
Glioblastoma 2	50.3 ± 12.9
Experiment B	
Control (DPBS)	13.3 ± 7.9
CSF (normal volunteer)	7.2 ± 5.5
CSF (sacral chordoma)	8.6 ± 5.1
CSF (malignant glioma)	5.1 ± 3.9
Cyst fluid (glioblastoma) 1	365.2 ± 80.6
Cyst fluid (glioblastoma) 2	643.3 ± 16.6

[a]The VPF activity for this series of Miles assays is expressed as mean counts per min per mg tissue ± S.E.M. Experiment A: VPF activity in conditioned medium from human tissue lines; experiment B: VPF activity in cerebrospinal fluid (CSF) and cystic glioblastoma fluid. DMEM, Dulbecco's modified Eagle's medium; DPBS, Dulbecco's phosphate-buffered saline. (Reprinted after modification from Bruce *et al.*,[14] with permission.)

or pepstatin A (an acid protease inhibitor which inactivates vascular permeability-inducing leukokinins). Treatment of HG-VPF with dithiothreitol abolished permeability-inducing activity, indicating the presence of at least one essential disulfide bond in this molecule. VPF displays a marked affinity for immobilized heparin (heparin-Sepharose, CL-6B). In addition, we found 90–95% binding of sample activity to hydrophobic resin (phenyl-Sepharose, CL-4B), and hydroxylapatite. While 40–45% of HG-VPF activity was bound

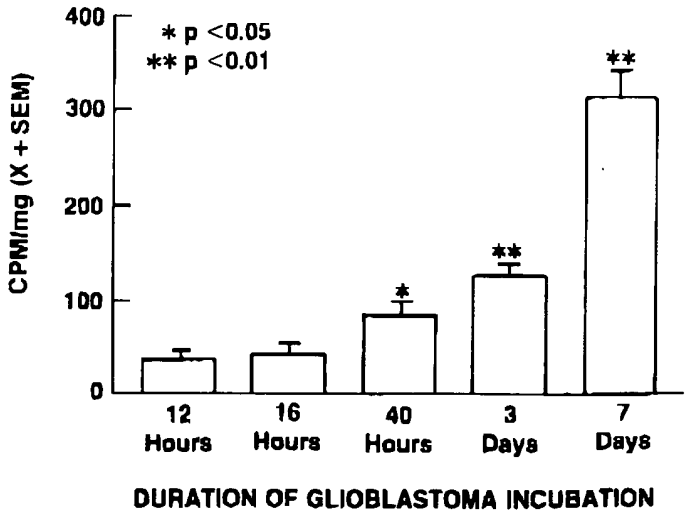

Figure 3. Histogram showing induction of vascular extravasation of ^{125}I-labeled bovine serum albumin, as a function of cell culture incubation time for glioblastoma line 1 samples. Vascular permeability factor (HG-VPF) activity is expressed as mean counts per minute (CPM)/mg + standard error of the mean (SEM). (Reprinted from Bruce *et al.*,[14] with permission.)

by negatively charged resin (carboxymethyl-Sepharose), only 5% of sample activity was bound to positively charged resin (diethylaminoethyl-Sephacel). The latter resin sequestered 80–85% of sample protein. Employing a heparin-Sepharose column and a NaCl gradient generator, it was determined that peak elution of HG-VPF activity occurs at a salt concentration of 0.45 N NaCl. Specific activity was increased 26-fold by this step. When this partially purified sample was applied to an HPLC sizing column, HG-VPF activity eluted in the 41- to 56-kDa fraction. Furthermore, an additional 25-fold increase in specific activity was realized with this step, thus increasing the total approximate purification to 1000-fold (Table 2).

C. Dexamethasone Inhibition of HG-VPF Expression and Activity[18]

Studies to determine how dexamethasone affects HG-VPF expression by cultured cells failed to show a direct toxic effect as measured by cell viability (> 98% by trypan blue exclusion), and final cell counts.[18] Although HG-VPF expression was significantly inhibited by the presence of glucocorticoid, this effect was not associated with a general inhibition of cellular protein synthesis by steroid (Table 3). We had previously determined that treatment of test animals with dexamethasone immediately before performing the permeability assay did not confer protection against HG-VPF-induced extravasation. Coinjection of dexamethasone with HG-VPF also failed to impart a membrane stabilizing protective effect as extravasation was unhindered. In fact, significant inhibition of HG-VPF activity *in vivo* only occurred in test animals given dexamethasone at least 1–2 hr before performing the permeability assay (Fig. 4). To determine whether the inhibitory action of dexamethasone was mediated by *de novo* protein synthesis, a series of *in vivo* studies were performed whereby actinomycin D was administered 2 hr prior to dexamethasone treatment. *In vivo* inhibition of HG-VPF activity by dexamethasone was found to be partially reversed in animals given actinomycin D prior to steroid. This suggested that *de novo* synthesis of a specific polypeptide intermediate is required for the protective effect of dexamethasone to

Table 2. Steps Required to Partially Purify Brain Tumor-Derived Vascular Permeability Factor[a]

Purification step	Volume (ml)	Total activity recovered	Protein activity	Specific purification	Degree of (-fold) recovery (%)	Activity
1. Conditioned serum-free medium	2000	9.78×10^6 dpm	200 mg (100%)	49 dpm/μg	—	100
2. Dialysis and lyophilization	30	9.92×10^6 dpm	60 mg (30%)	165 dpm/μg	3.4×	101
3. Heparin affinity	250	1.59×10^6 dpm	375 μg (2%)	4,244 dpm/μg	87×	16
4. Ultrafiltration	2.5	3.90×10^6 dpm	<25 μg (0.01%)	15,600 dpm/μg	318×	4
5. HPLC (TSK2000)	1.0	9.16×10^6 dpm	<20 μg (0.01%)	45,800 dpm/μg	935×	9.4

[a]Reprinted after modification from Criscuolo *et al.*,[18] with permission.

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Table 3. Effect of Dexamethasone on VPF Expression, Cell Number, Cell Viability, and Protein Synthesis[a,b]

Period of incubation	Dexamethasone	VPF activity[c]	Cell number/well[d]	Protein synthesis[e]
2 days	0 (control)	100%	6.59 ± 0.026	154 ± 6
	10^{-11} M	100%	6.23 ± 0.156	163 ± 7
	10^{-9} M	88%	6.75 ± 0.243	168 ± 13
	10^{-7} M	54%	6.83 ± 0.157	165 ± 7
	10^{-5} M	28%	7.39 ± 0.266	162 ± 6
4 days	0 (control)	100%	6.81 ± 0.620	130 ± 18
	10^{-11} M	70%	7.53 ± 0.610	140 ± 17
	10^{-9} M	69%	7.32 ± 0.264	122 ± 14
	10^{-7} M	68%	8.64 ± 0.164	99 ± 5
	10^{-5} M	54%	7.77 ± 0.303	134 ± 4
7 days	0 (control)	100%	6.56 ± 0.289	126 ± 7
	10^{-11} M	94%	6.62 ± 0.384	131 ± 7
	10^{-9} M	100%	6.55 ± 0.341	138 ± 4
	10^{-7} M	53%	6.96 ± 0.714	129 ± 5
	10^{-5} M	48%	7.55 ± 0.136	118 ± 4

[a]Reprinted after modification from Criscuolo *et al.*,[18] with permission.

[b]Cell viability was > 98% by trypan blue exclusion. Values are means ± S.E.M.

[c]The S.E.M. for VPS activity measurements was determined to be 7.5%. The control VPS activity values (0 dexamethasone) for days 2, 4, and 7 were 1294, 7677, and 11,555 dpm, respectively.

[d]Cell counts were performed in quadruplicate using a Coulter counter and are expressed as multiples of 10^5 cells.

[e]Protein synthesis is expressed as mean picomoles of [^{3}H]leucine incorporated per hour per milligram protein ± S.E.M. for four samples.

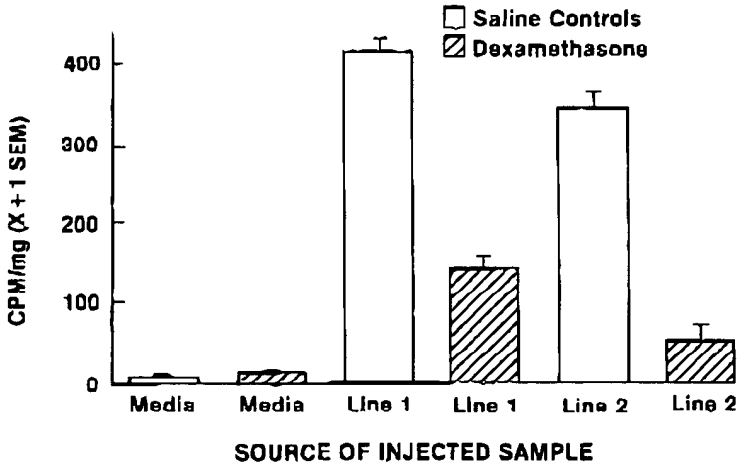

Figure 4. Histogram showing the inhibitory influence of dexamethasone on the *in vivo* activity of vascular permeability factor (HG-VPF) derived from glioblastoma multiforme lines 1 and 2. These findings were determined by the Miles assay. The VPF activity is expressed as mean counts per minute (CPM)/mg + 1 standard error of the mean (SEM). (Reprinted from Bruce *et al.*,[14] with permission.)

occur, thereby strongly arguing against nonspecific steroid-induced membrane stabilization as the key physiological event (Table 4).

D. HG-VPF Effect on Endothelial Cell Cytosolic Calcium Ion Concentration[19]

Recent advances in our understanding of endothelial cell growth requirements have allowed their routine culture for experimental purposes.[5,9,10,20,32,45,46,103] Endothelial cells derived from brain capillaries have received considerable attention because of their unique barrier characteristics.[5,9] Cytosolic free Ca^{2+} plays a pivotal role in endothelial cytoskeletal alterations and subsequent microvascular extravasation.[22,39,52,72,82] Studies of cytosolic calcium changes have been greatly facilitated by the development of a series of novel fluorescent calcium ion probes (quin-2/AM, indo-2/AM, fura-2/AM, fura-3/AM) possessing molecular structures similar to EGTA.[37,60,92] They differ from EGTA in that they contain aromatic rings capable of electrostatic interactions with the functional groups that participate in the chelation of free cytosolic calcium ions $[Ca^{2+}]_i$ Chelation of divalent cations changes the fluorescence and ultraviolet light-absorption properties of these molecules. Alterations in fluorescence are in turn detected and quantitated by a spectrofluorometer. Fura-2/AM has proven particularly useful with its high sensitivity and specificity for calcium ions. Because of its lipophilicity, fura-2/AM is rapidly internalized by endothelial cells. Once internalized, however, deesterification of the acetoxy-methyl group (fura-2/AM → fura-2) converts it to a lipophobic free acid, with only minimal capability for diffusion out of the cell. Cytosolic calcium ion changes in the nanomolar range are readily detectable with this probe. Partially purified HG-VPF has been shown to induce significant calcium ion transients in several varieties of endothelial cells sustained in monolayer cultures.[19] It did not, however, elicit a calcium response in two nonendothelial cell lines (U251 glioma and fibroblasts). The endothelial cytosolic calcium changes were typically rapid or only slightly delayed in onset (15–45 sec), and varied in magnitude depending upon the cell type being studied (Fig. 5). The largest responses were observed in human endothelial cells. It

Table 4. Effect of Actinomycin D on Dexamethasone-Induced Inhibition of Vascular Permeability Factor Activity[a]

Experimental group (20 animals)	[125I]-BSA extravasation (mean ± S.E.)		% inhibition by dexamethasone	
	Brain tumor VFP	Histamine	Brain tumor VPF	Histamine
Control ($n = 5$)	22,000 ± 2,427	7977 ± 1005	0%	0%
Dexamethasone ($n = 5$)	3,160 ± 529	2357 ± 314	86%	71%
Actinomycin D ($n = 5$)	11,412 ± 860	1623 ± 138	0%	0%
Actinomycin D + dexamethasone ($n = 5$)	6,976 ± 878	585 ± 193	39%	64%

[a]Reprinted after modification from Criscuolo *et al.*,[18] with permission.
$p < 0.0005$ (control versus dexamethasone).
$p = 0.006$ (dexamethasone versus actinomycin D + dexamethasone).
$p = 0.001$ (control versus dexamethasone).
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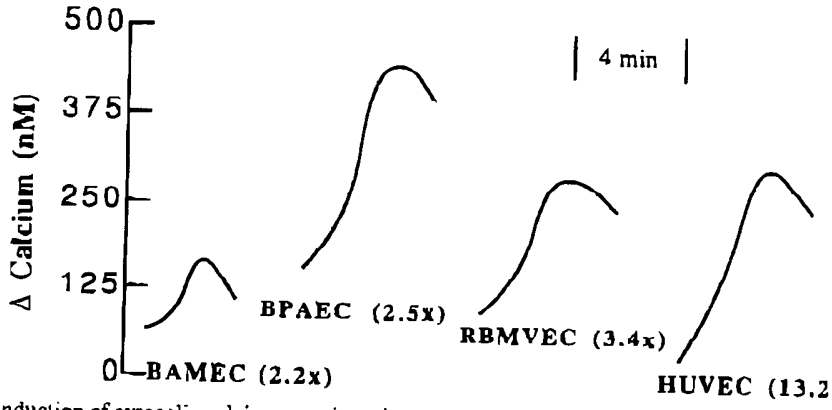

Figure 5. Induction of cytosolic calcium transients in a variety of endothelial cell lines by partially purified human malignant glioma-derived vascular permeability factor (HG-VPF). The relative degree of intracellular calcium change induced by a standard HG-VPF stimulus is indicated in parentheses. All graphic data were obtained by computerized tracing of original hard-copy data after reassignment of *y*-axis values by conversion to absolute Ca^{2+} concentrations. BAMEC, bovine adrenal medullary endothelial cells; BPAEC, bovine pulmonary artery endothelial cells; RBMVEC, rat brain microvessel endothelial cells; HUVEC, human umbilical vein endothelial cells. Note that the largest calcium responses to HG-VPF occurred in human venous endothelial cells. The possible significance of this finding is discussed in the text. (Reprinted from Criscuolo *et al.*,[19] with permission.)

has already been postulated that such variations may relate to some degree of molecular species-specificity despite the highly conserved nature of VPF activity.[79] Peak HG-VPF-induced intracellular calcium ion elevations were observed within 60 sec, exhibited a dose–response phenomenon, and were followed by a sustained elevation above baseline for 5 to 10 min thereafter (Fig. 6). In contrast, exposure of the endothelial cells to the flow-through product of HG-VPF containing glioma-conditioned medium after binding of all HG-VPF activity to a heparin-Sepharose affinity column, did not produce a change in intracellular calcium. The brief time course of the HG-VPF-induced cytosolic calcium changes *in vitro* is compatible with the *in vivo* kinetics, and is supported by other data showing prolonged stimulus–response coupling to occur after only transient intracellular calcium elevations.[72]

Exposure of endothelial cell suspensions to elevated extracellular potassium chloride (120 mM KCl) failed to induce cytosolic calcium transients. Furthermore, verapamil (10 μM) failed to inhibit HG-VPF-induced calcium changes. This finding suggests that HG-VPF increases cytosolic calcium by a route other than verapamil-sensitive voltage-gated calcium channels (Fig. 7). Addition of 2 mM lithium, cobalt, manganese, or lanthanum ions inhibited HV-VPF-induced calcium transients (Fig. 8), as did the absence of extracellular calcium after chelation with EGTA. This indicates that HG-VPF-induced calcium ion changes are largely dependent upon the influx of extracellular calcium $[Ca^{2+}]_e$ through membranous calcium channels. Finally, incubation of endothelial cells for 2 hr with 10 μM dexamethasone before exposure to HG-VPF-containing medium resulted in inhibition of the HG-VPF-induced cytosolic calcium changes (Fig. 9). This inhibition was not observed in similarly treated cells exposed to ATP, nor did it occur when cells were incubated with dexamethasone for less than 1 hr; suggesting that the steroid-induced inhibition was specific for HG-VPF-induced calcium flux, and that dexamethasone may mediate its effect through an intermediary requiring *de novo* synthesis.

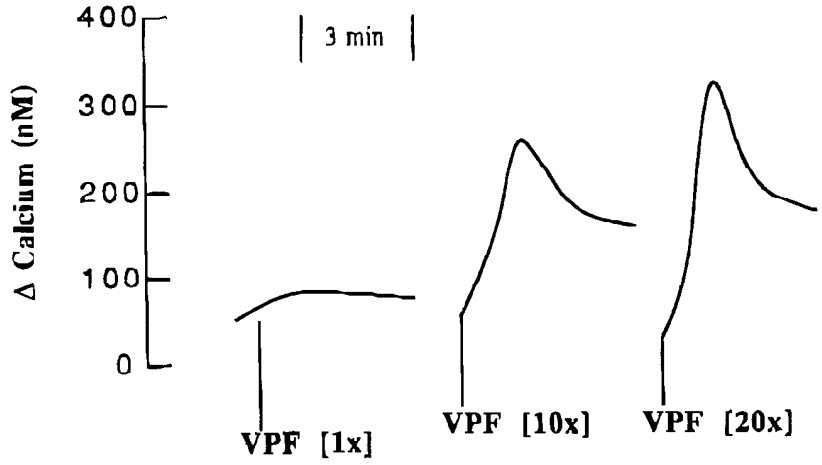

Figure 6. Dose-response relationship between human malignant glioma-derived vascular permeability factor (HG-VPF) stimulus and cytosolic calcium transients induced in human umbilical vein endothelial cells. Fold concentration of HG-VPF is expressed as multiples of a standard solution containing 0.25 mg/ml (1×) of partially purified lyophilized glioma-conditioned medium dissolved in Dulbecco's phosphate-buffered saline (2.5 mg/ml = 10×, 5.0 mg/ml = 20×). (Reprinted from Criscuolo *et al.*,[19] with permission.)

Another polycationic substance (protamine), known to induce changes in vascular permeability,[87,97,98] was also shown to induce physiological changes in endothelial intracellular calcium, when present in concentrations as low as 10 μg/ml. Induction of calcium transients by protamine was rapid in onset, peaked within 30 sec, and sustained a level above baseline for several minutes thereafter. The presence of extracellular divalent cations (2 mM cobalt or manganese) reduced peak $[Ca^{2+}]_i$, but also more completely affected the

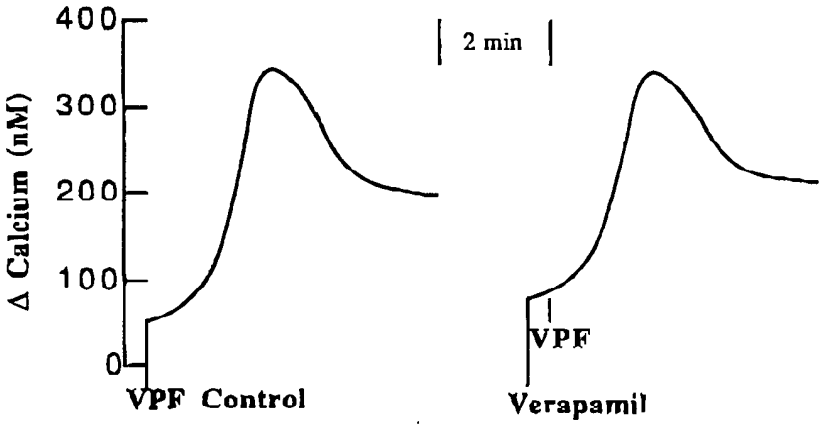

Figure 7. Failure of verapamil to inhibit human glioma-derived vascular permeability factor (HG-VPF)-induced calcium transients. Prior exposure of endothelial cells to 10^{-6} M verapamil did not diminish cytosolic calcium changes induced by HG-VPF. Calcium transients were not induced by exposure of endothelial cells to 120 mM KCl (data not shown). This suggests that HG-VPF-induced influx of $[Ca^{2+}]_e$ occurs by a mechanism other than voltage-gated calcium ion channels. (Reprinted from Criscuolo *et al.*,[19] with permission.)

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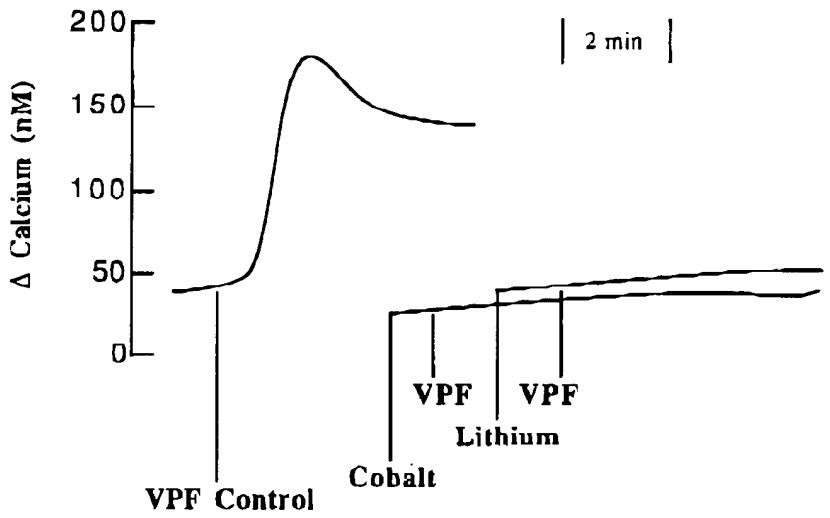

Figure 8. Inhibition of human glioma-derived vascular permeability factor (HG-VPF)-induced calcium ion transients by 2 mM Li^+ and Co^{2+} cations. Inhibition also occurred with 2 mM concentrations of Mn^{2+} and La^{3+} cations (data not shown). Nonspecific cationic calcium channel blockers appear to inhibit HG-VPF-induced intracellular calcium ion transients. Similarly, chelation of extracellular calcium ions with EGTA partially inhibited induction of calcium transients by HG-VPF (data not shown). This finding suggests that influx of extracellular calcium via non-voltage-gated membranous channels may be the primary event in the HG-VPF-elicited response. There is some indication that mobilization of sequestered intracellular calcium stores $[Ca^{2+}]_{is}$ may also play a role as a small but significant calcium transient was generated despite complete chelation of extracellular calcium by EGTA (data not shown). (Reprinted from Criscuolo *et al.*,[19] with permission.)

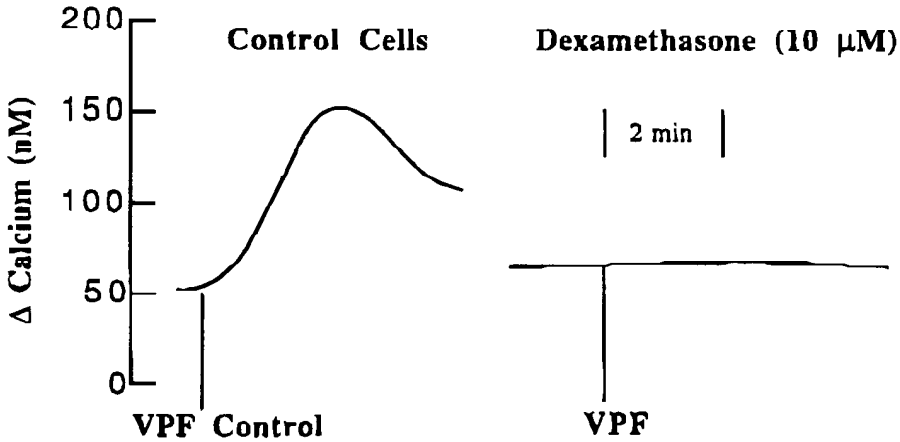

Figure 9. Inhibition of human glioma-derived vascular permeability factor (HG-VPF)-induced cytosolic calcium ion transients in endothelial cells incubated 2 hr with 10 μM dexamethasone. The dose of dexamethasone used is consistent with actual brain tumor tissue levels of dexamethasone determined from neurosurgical biopsy specimens. This finding supports *in vivo* findings that dexamethasone will inhibit microvascular permeability induced by HG-VPF when test animals are given a steroid dose 1–2 hr before injection of HG-VPF samples. (Reprinted from Criscuolo *et al.*,[19] with permission.)

postmaximum levels resulting in a rapid downslope and normalization of intracellular calcium. These findings indicated that combined mobilization of intracellular calcium stores (rapid upslope and peak levels), as well as influx from the extracellular compartment (peak and sustained levels), are involved in the response to HG-VPF and protamine. It also suggests that neutralization of endothelial cell surface charge (anionic) by polycationic substances (VPFs, protamine, angiogenesis factors) may play a role in mediating the actions of these substances. This is supported by ultrastructural evidence that exposure of renal glomeruli to protamine results in disorganization of the epithelial cell foot process architecture (podocytes) investing the microvascular glomeruli, and subsequent leakage of 2protein into the urine. Endothelial surface charge neutralization by polycations may simply climinate the electrostatic barrier that exists to the vast majority of proteins (negatively charged), allowing them to escape through pores in fenestrated vessels.

VI. SOME THOUGHTS ON THE UNIQUE RELATIONSHIP BETWEEN VPFs, THE BRAIN TUMOR MICROVASCULATURE, DEXAMETHASONE, AND NEOPLASTIC VASOGENIC BRAIN EDEMA

The microvasculature of brain tumors displays several distinctive ultrastructural features that allow ready distinction from normal cerebral microvessels. The presence of widened endothelial cell junctions, discontinuous tight junctions, cellular membrane fenestrations, noncontiguous basement membranes, active micropinocytosis, and paucity of mitochondria, sharply contrast with normal blood–brain barrier architecture.[1,12,36,55–57,64] These features, which also typify endothelium lining the normal peripheral vasculature, have previously been associated with the water and protein extravasation, and consequent cerebral edema that occurs with malignant brain tumors.[13,48,105] Like blood–brain barrier microvessels, however, the normal peripheral vasculature is not inherently permeable to macromolecules. Nevertheless, tumors occurring outside the CNS also exhibit microvascular extravasation that is clinically manifested as malignant effusions, ascites, and tissue edema.[1,78,86,93]

The normal peripheral vasculature is exquisitely sensitive to permeability induction by histamine, bradykinin, serotonin, prostaglandins, and a variety of other physiological substances.[62,65] In contradistinction, normal cerebral microvessels are totally incapable of responding to these substances when tested *in situ*. Similarly, HG-VPF, which clearly is active in the guinea pig and rat cutaneous microvasculature, failed to evoke extravasation of fluorescein–albumin, [^{125}I]-bovine serum albumin, or edema fluid as evidenced by tissue specific gravity determinations, when injected into normal rat brain (unpublished observations of G. R. Criscuolo and E. H. Oldfield). However, injection of HG-VPF into C6 gliomas that had been implanted into rat brains showed brain tumor microvessels to be capable of responding with increased permeability *in situ* (unpublished observations of G. R. Criscuolo and E. H. Oldfield).

Vascular endothelial cells from peripheral and CNS sources are known to possess a complex cytoskeletal architecture.[22,82] The endothelial cytoskeleton is composed of actin, myosin, and tropomyosin proteins that serve to regulate cellular configuration. Therefore, cellular mobility and vascular permeability are processes that are likely to result from

changes in intracellular calcium ions that occur in response to angiogenesis factors and VPFs. Stimuli that induce cytosolic calcium transients and change endothelial cell shape, ultimately result in opening of interendothelial junctions at the level of the postcapillary venule, which in turn results in vascular extravasation.[52,63,85,89] Histamine-induced cytosolic calcium changes have been shown to correlate with changes in endothelial F-actin content, and passage of albumin across endothelial monolayers derived from human umbilical veins.[72] In fact, many of the commonly recognized mediators of microvascular permeability have been shown to induce transient elevations in endothelial cytosolic calcium, irrespective of the variety of endothelial cells tested.[19,31,35,52,60,72,89] Significantly, normal brain microvascular endothelial cells are equally capable of responding with calcium ion transients, and, by conjecture, cytoskeletal alterations that would culminate in vascular extravasation. These findings, and the morphological similarities between brain tumor vessels and the normal peripheral vasculature, suggest an alternative hypothesis for the induction of vascular permeability in brain tumors.

Perhaps the reason blood–brain barrier microvessels are incapable of responding to permeability mediators *in situ*, solely relates to their interendothelial junctions being physically joined together by continuous pentalaminar tight junctions. The extreme integrity of this junction is supported by experimental studies of osmotic disruption of the blood–brain barrier by mannitol, wherein investigators showed the interendothelial clefts to be closed, and the tight junctions to be continuous and intact after exposure to that potent physical agent: opening of the barrier by mannitol was associated with an increased transgression of micropinocytotic vesicles between the luminal and abluminal endothelial surfaces.[24] Further support derives from the knowledge that peritumoral brain edema emanates directly from the tumor bed, and is not the result of HG-VPF-induced extravasation in the surrounding blood–brain barrier microvessels.[105] Therefore, one may reasonably put forth the hypothesis that an alteration or absence of such a critical component of blood–brain barrier as the interendothelial tight junction may at once render brain tumor microvessels similar to the peripheral microvasculature, both anatomically and with respect to their ability to respond to permeability mediators. As a result, brain tumor microvessels would be capable of responding to the HG-VPF secreted by surrounding tumor cells, and production of vasogenic cerebral edema would result.

Although the study of VPFs has already yielded valuable insight into a mechanism of tumor-mediated vascular permeability, many questions remain unanswered. Several questions specifically pertinent to neoplastic brain edema immediately arise: Why does peritumoral brain edema not follow an even more malignant course? If brain tumor microvessels remain constantly exposed to HG-VPF, and respond with a constant rate of plasma extravasation, why would a patient not succumb to a rampant increase in ICP within perhaps minutes, hours, or a few days at most? This may be partly explained by known mechanisms of brain edema resolution. Excess extracellular fluid normally travels through the brain interstitium in a centripetal direction (toward the CSF-containing ventricular system) by a passive process referred to as bulk flow. In addition, astrocytic cells are known to be actively involved in the imbibition of excess tissue fluid. This issue may also be partly explained by observations indicating that HG-VPF is capable of inducing a period of unresponsiveness, refractoriness, or tachyphylaxis, whereby consecutively applied stimuli will not result in cytosolic calcium changes or further vascular extravasation.[14,18,19,78,79]

Supporting this notion is the common occurrence of a period of relative unresponsiveness (refractory period) in many physiological cascades, as well as in a variety of physiologically excitable cells (neurons, photoreceptor cells, muscle cells).

The clinical efficacy of dexamethasone in the setting of peritumoral brain edema has been well described and documented.[29,42,48,54,69,104,106] We have been able to demonstrate several levels at which dexamethasone may specifically exert its beneficial effects in this focused pathophysiological setting.[14] Concentrations of dexamethasone that occur in our patients' brain tumors will specifically suppress production of HG-VPF by cultured human malignant glioma cells.[18] This finding alone suggests a powerful mechanism for steroid efficacy. In addition, a distinct and separate mechanism of steroid action may occur at the level of the key biological component for this process; the microvascular endothelial cell. This is supported by the finding of calcium transient suppression by dexamethasone in cultured endothelial cells.[19] Lastly, a glimpse into the mechanism by which dexamethasone affects target cells (glioma cells and endothelial cells) is provided by the following findings: (1) Coinjection of HG-VPF with dexamethasone did not alter the extent of vascular extravasation.[14] (2) Pretreatment of test animals with dexamethasone 1–2 hr before HG-VPF injection substantially inhibited vascular extravasation whereas pretreatment less than 1 hr before resulted in no inhibition of HG-VPF activity.[14,18] (3) Pretreatment of test animals with actinomycin D before dexamethasone exposure, resulted in significant, albeit slightly reduced, HG-VPF-induced extravasation.[18] The implication is that dexamethasone exerts its actions indirectly, by inducing *de novo* synthesis of a polypeptide intermediary, rather than by nonspecific membrane stabilization. This concept has been put forth as an explanation for the protective effect of dexamethasone in rats affected by global cerebral ischemia.[90] Furthermore, a second messenger polypeptide (variably referred to as "macrocortin," "lipocortin," "endocortin," or "renocortin") has been identified, characterized, and found to mediate the effect of glucocorticoids by inhibition of phospholipase A_2.[7] Induction of macrocortin synthesis requires steroid receptor occupation and *de novo* protein synthesis. It therefore appears possible that HG-VPF may act by inhibiting a rate-limiting enzyme in the prostaglandin cascade (phospholipase A_2).[18,19] This is corroborated by the work of others who have shown prostaglandins to be potent inducers of cytosolic calcium transients in endothelial cells.[30,31]

Additional investigations using a completely purified VPF product are clearly warranted, and should better define the kinetics, refractoriness to sequential stimulation, and dose responsiveness of VPF-induced microvascular extravasation. Furthermore, comparison of endothelial cells derived from different anatomical sources (brain, retina, lung, adrenal, umbilical cord), from differing sized vessels (arterial, arteriolar, capillary, venous, venular), and from several species (human, bovine, rodent), should determine the extent, and degree of specificity of HG-VPF activity with regard to these variables.[5,9,10,20,32,45,46,61,74,75,99] Information to date strongly favors a direct action of HG-VPF on the vascular endothelial cell. The rapid and reversible kinetics of HG-VPF activity *in vivo* and *in vitro*, its remarkable affinity for immobilized heparin, and its ability to induce significant changes in endothelial cytosolic calcium lend solid support to, if not affirmation of, this premise.[14,18,19] A well-defined negatively charged glycocalyx, composed primarily of sulfated glycosaminoglycans, is present on capillary endothelial cell surfaces.[2] This cell coat is largely composed of polyanionic heparan sulfate and sialic acid residues. Its primary function is thought to be the selective regulation of endothelial cell binding and transcapil-

lary passage of macromolecules. The cationic nature of the HG-VPF molecule at physiological pH would therefore suggest a means for direct, albeit nonspecific, electrostatic attraction of the HG-VPF molecule to the endothelial cell surface. This in turn would likely facilitate the subsequent binding and interaction between the active site on the HG-VPF molecule and a specific membrane receptor.

The investigations outlined herein support an important role for HG-VPF in the expression of peritumoral brain edema. In addition, an explanation for the efficacy of dexamethasone in the treatment of symptomatic brain edema associated with cerebral neoplasms is strongly suggested. The clinical and experimental findings are also supported by the occurrence of steroid receptors in cerebral tumors,[33,49,104,106] and the lack of steroid efficacy in the setting of ischemic, toxic, hemorrhagic, and traumatic cerebral edema.[23,38,67] Inhibition of HG-VPF activity by glucocorticosteroids such as dexamethasone appears to be related to highly specific actions at the cellular level (i.e., inhibition of tumor cell expression of VPF, and inhibition of VPF-induced endothelial cell calcium responses). Perhaps a better understanding of the subcellular mechanisms involved in steroid-induced HG-VPF inhibition will suggest either worthwhile refinements in our use of these agents, or the means to develop novel methods that are safer and more effective in the treatment of patients with neoplastic vasogenic brain edema.

VII. CLOSING REMARKS

The magnitude of the brain tumor problem has been outlined. Some patients with malignant brain tumors have enjoyed extended functional survival relating largely to aggressive surgical excision, radiotherapy, and chemotherapy. Certain brain tumors (meningiomas) are considered histologically and biologically benign, and therefore ostensibly curable. Nevertheless, a great deal of perioperative neurological morbidity has been associated with excision of these lesions, and largely relates to vasogenic edema production by some meningiomas. One of the most significant therapeutic advances in neuro-oncology in the past two decades has been the recognition of the efficacy of corticosteroids in the symptomatic treatment of patients with malignant brain tumors. Their salutory effects on intracranial hypertension have resulted in better tolerance of both surgical therapy and radiotherapy. However, the extended use of high-dose corticosteroids is not without complications. Consequences of their long-term use include sodium and water retention, hypertension, diabetes mellitus, sepsis related to impaired immune function, connective tissue alterations, gastrointestinal ulceration, aseptic necrosis of the femur head, and altered mentation including mania, psychotic depression, and euphoria. Investigation into the precise mechanism by which steroids act upon peritumoral cerebral edema may eventually provide clinicians with safer and more effective therapeutic alternatives for patients afflicted with brain tumors.

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